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Involvement of the NO/cGMP/K_{ATP} pathway in the antinociceptive effect of the new pyrazole 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-021)

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ABSTRACT

The pyrazol compounds are known to possess antipyretic, analgesic and anti-inflammatory activities. This study was conducted to investigate the peripheral antinociceptive effect of the pyrazole compound 5-(1-(3-Fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-021) and involvement of opioid receptors and of the NO/cGMP/K_{ATP} pathway. The oral treatments in mice with LQFM-021 (17, 75 or 300 mg/kg) decreased the number of writhing. In the formalin test, the treatments with LQFM-021 at doses of 15, 30 and 60 mg/kg reduced the licking time at both neurogenic and inflammatory phases of this test. The treatment of the animals with LQFM-021 (30 mg/kg) did not have antinociceptive effects in the tail-flick and hot plate tests. Furthermore, pre-treatment with naloxone (3 mg/kg i.p.), L-name (10 mg/kg i.p.), ODQ (10 mg/kg i.p.) or glibenclamide (3 mg/kg i.p.) antagonized the antinociceptive effect of LQFM-021 in both phases of the formalin test. In addition, it was also demonstrated that the treatments of mice with LQFM-021(15, 30 and 60 mg/kg) did not compromise the motor activity of the animals in the chimney test. Only the highest dose used in the antinociceptive study promoted changes in the open field test and pentobarbital-induced sleep test, thus ruling out possible false positive effects on nociception tests. Our data suggest that the peripheral antinociception effects of the LQFM-021 were mediated through the peripheral opioid receptors with activation of the NO/cGMP/K_{ATP} pathway.

1. Introduction

Pain is one of the most prevalent facts that limits productivity and reduce the quality of life. Although there are many effective analgesics, there is some concern regarding their safety and side-effects. The identification of compounds that can treat the pain with low induction of side-effects remains a major challenge in research [1,2].

Nitric oxide (NO) is known as an important signaling molecule regulating a wide range of physiological, biochemical and molecular processes [3]. The NO has a role in synaptic transmission in both the central nervous system (CNS) and peripheral. Some studies have been associated nitric oxide with antinociceptive effect [4,5]. The synthesis of the NO is mediated for different isoforms of nitric oxide synthase (NOS), which enzymatically converts *L*-arginine to *L*-citruline and NO [6]. After its formation, NO activates the guanylate cyclase enzyme, which is responsible for the increase in intracellular levels of cGMP resulting in peripheral antinociceptive effect [4,7].

Works have been showed that opioid receptor agonist induces peripheral antinociception, which was associated with activation of the NO/cGMP pathway [4,8]. The peripheral antinociceptive effects of various drugs have been implicated in the activation of the NO/cGMP/K_{ATP} channel pathway, such as morphine [4,9], dipyrone [10], diclofenac [11] and xylazine [12].

The agents that promote the release of nitric oxide, in the periphery, antagonize the hyperalgesia induced by inflammatory stimuli and phosphodiesterase inhibitors, enzymes that inactivate cGMP, potentiate the analgesic effects of these drugs [13,14].

Pyrazole compounds have been reported that have several biological activities such as antimicrobial, antineoplastic, anti-inflammatory, antipyretic and antinociceptive activities [15]. In this context, new pyrazole derivatives are reported by their antinociceptive, anti-inflammatory and antipyretic effects in different animal models [16–21].

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Martins et al. [22] showed that a new synthetic derivative of pyrazole (LQFM021) is a possible PDE-3 inhibitor and has vasorelaxant activity and low toxicity. The aim of this work was to evaluate the antinociceptive effect of a new pyrazole compound, LQFM-021, and investigate the involvement of the opioid receptor and NO/cGMP/ATP-sensitive K^+ channel pathway in this effect.

2. Materials and methods

2.1. Structure of LQFM021

The compound 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-021) was synthesized in to "Laboratório de Química Farmacêutica Medicinal" (LQFM), Faculty of Pharmacy, Federal University of Goiás, according to synthetic route described by Martins et al. [22]. The chemical structure of compound LQFM-021 is shown in Fig. 1.

2.2. Animals

Experiments were performed using male Swiss albino mice (25–30 g) from the Central Animal House of the Federal University of the State of Goiás (UFG). Animals were kept in plastic cages at 22 ± 2 °C with free access to pellet food and water and on a 12 h light/dark cycle, in compliance with the International Guiding Principles for Biomedical Research Involving Animals. The animals were acclimatized for 7 days before the beginning of the experiments. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal Experimentation. The experimental protocols were approved by the Ethic Commission of UFG (number: 17/13).

2.3. Drugs and chemicals

The chemicals used in this study were LQFM-021, synthesized in to "Laboratório de Química Farmacêutica Medicinal" (LQFM) (Faculty of Pharmacy, Federal University of Goiás), acetic acid (Merck, USA), DMSO (Sigma Chemical, USA); formaldehyde (Synth, Brazil), glibenclamide (Sigma-Aldrich, St. Louis, MO, USA), indomethacin (Indocid®, Merck Sharp & Dohme Farmac; Goiarmacê), *L*(+)-arginine (Acrôs organics, EUA), N^G-nitro-*L*-arginine methyl ester (*L*-name) (Cayman Chemical Company, USA), Morphine hydrochloride (Dimorf®, Cristalia, SP, Brazil), naloxone chloridrate (Narcan®, Cristalia, SP, Brazil), 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ) (Sigma-Aldrich, St. Louis, MO, USA), pentobarbital (Abbott, Brazil); LQFM-021 was dissolved in 10% DMSO in saline and all other drugs were dissolved in 0.9% saline.

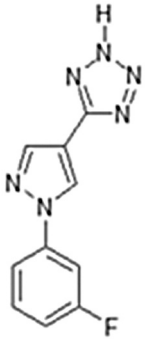

Fig. 1. Chemical structure of compound LQFM-021: 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole.

2.4. Nociceptive tests

2.4.1. Acetic acid-induced abdominal writhing test

The acetic acid-induced nociception was performed as described previously by Koster et al. [23]. Groups of mice (n = 8) were treated by gavage (p.o.) with vehicle (10% DMSO 10 mL/kg), LQFM-021 (17, 75 and 300 mg/kg) or indomethacin (10 mg/kg, positive control for antinociceptive activity) 60 min before the application of acetic acid solution (1.2% v/v; 10 mL/kg, i.p.). The number of abdominal constrictions (writhing) was counted for each animal, over a period of 30 min after acetic acid injection, and the results are expressed as the means ± SEM of number of writhing.

2.4.2. Formalin test

The formalin-induced nociception was performed as described previously by Hunskaar et al. [24]. Groups of mice (n = 8) were treated by gavage (p.o.) with vehicle (10% DMSO 10 mL/kg), LQFM-021 (7.5, 15, 30 and 60 mg/kg) or indomethacin (10 mg/kg – positive control for antinociceptive activity in the second phase) or morphine (5 mg/kg s.c. – positive control for antinociceptive activity in the first and second phase). Following sixty minutes after treatment by gavage (p.o.) or thirty minutes by (s.c.) was administrated of 20 μL of 3% formalin (in saline) into the plantar surface of the right hind paw. After phlogistic agent injection, the mice were placed into an acrylic box and a mirror was placed under the boxes to enable unhindered observation of the formalin-injected paw for 30 min. Pain reaction time (licking time) was assessed during two periods, 0–5 min, the first phase (neurogenic pain caused by direct stimulation of the nociceptors), and from 15 to 30 min, the second phase (inflammatory pain caused by release of inflammatory mediators). These results are expressed as the means ± SEM in seconds.

2.4.3. Tail flick test

The tail flick test was performed as described previously by D'Amour and Smith [25]. The time taken to flick the tail (latency) when the tail was exposed to a heat source, using the analgesimeter. The animals were divided into three experimental groups (n = 8) consisting of animals treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM-021 (30 mg/kg, p.o.) or morphine (5 mg/kg, s.c. – positive control for antinociceptive activity). The latency to pain reaction was measured at –30, 0, 30, 60, 90 and 120 min after treatment. A cut-off of 15 s was set. This test was conducted using an analgesimeter (Insight). The results are expressed as means ± SEM in seconds at the different times.

2.4.4. Hot plate test

The hot plate test was performed as described previously by Woolfe and MacDonald [26]. The hot-plate test is a model for studying supraspinally acting analgesic compounds. The latency (in seconds) to the reaction of the mice to thermal stimuli, as expressed by licking, shaking or lifting of the hind paws, on a hot-plate maintained at 55.5 ± 0.5 °C. The animals were divided into three experimental groups (n = 8) consisting of animals treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM-021 (30 mg/kg, p.o.) or morphine (5 mg/kg, s.c. – positive control for antinociceptive activity). The latency to pain reaction was measured at –30, 0, 30, 60, 90, 120 and 150 min after treatment. A cut-off of 20 s was set. This test was conducted using a hot plate (Insight). The results are expressed as means ± SEM in seconds at different times.

2.5. Analysis of the possible mechanism action

2.5.1. Involvement of opioid receptors

To evaluate the involvement of opioid receptors in the antinociceptive effect of LQFM-021 in the formalin test, the mice (n = 8) were pre-treated with saline (10 mL/kg i.p.) or (naloxone,

3 mg/kg i.p. – non-selective opioid antagonist) 15 min before of the treatment with vehicle (10% DMSO 10 mL/kg), LQFM-021 (30 mg/kg, p.o.), indomethacin (10 mg/kg p.o.) or morphine (5 mg/kg s.c.). Following sixty minutes after treatment by gavage (p.o.) or thirty minutes by subcutaneous administration (s.c.) the animals received formalin (3% v/v) and the experiment proceeded as discussed in section 2.4.2. [24,27].

2.5.2. Involvement of the NO pathway

To investigate the role of nitric oxide in the antinociceptive effect of LQFM-021 in the formalin test, the mice (n = 8) were pretreated with *L*-name (10 mg/ kg, i.p. – NO synthase inhibitor) or saline (10 mL/kg i.p.) after 15 min, were treated with vehicle (10 mL/kg p.o.), LQFM-021 (30 mg/kg, p.o.), *L*-arginine (600 mg/kg, i.p.) or morphine (5 mg/kg s.c.). Following sixty minutes after treatment by gavage (p.o.) or thirty minutes by (i.p.), the animals received formalin (3% v/v) and the experiment proceeded as discussed in section 2.4.2. As described previously with slight modifications by Perimal et al. [28] and Mansouria et al. [29].

2.5.3. Involvement of cyclic guanosine monophosphate (cGMP) pathway

To assess the possible involvement of cGMP in the antinociceptive effect of LQFM-021 in the formalin test, the mice (n = 8) were pretreated with saline (10 mL/kg i.p.) or ODQ (10 mg/kg i.p. – guanylyl cyclase inhibitor) 15 min before treatment with vehicle (10 mL/kg p.o.) or LQFM-021 (30 mg/kg, p.o.). Following sixty minutes after treatment by gavage (p.o.) the animals received formalin (3% v/v) and the experiment proceeded as discussed in section 2.4.2. As described previously with slight modifications by Perimal et al. [28] and Mansouria et al. [29].

2.5.4. Involvement of ATP-sensitive K^+ channel

To investigate the role of K^+ channel in the antinociceptive effect of LQFM 021 in the formalin test, the mice (n = 8) were pretreated with saline (10 mL/kg i.p.) or glibenclamide (10 mg/kg, i.p. – specific K^+_{ATP} blocker channel) 15 min before treatment with vehicle (10 mL/kg p.o.) or LQFM-021 (30 mg/kg, p.o.). Following sixty minutes after treatment by gavage (p.o.) the animals received formalin (3% v/v) and the experiment proceeded as discussed in section 2.4.2. As described previously by Perimal et al. [28] and Mansouria et al. [29], with slight modifications.

2.6. Evaluation of behavioral alterations

2.6.1. Chimney test

The chimney test permits the detection of muscle relaxing agents and/or drugs that produce motor incoordination. Briefly, after 60 minutes of treatment with 10 mL/kg vehicle (10% DMSO 10 mL/kg p.o.), LQFM-021 (15, 30 and 60 mg/kg, p.o.) or after 30 minutes of treatment with morphine (5 mg/kg s.c.), the animals were (n = 8) placed in the tube (25 cm length, 3 cm diameter) with a rough inner surface. Motor impairments were indicated by the animals' inability to climb backwards up a tube at time of 30 seconds. The results are expressed as the means ± SEM, as described by Coleta et al. [30].

2.6.2. Open field test

The open field test assessed mice ambulatory behavior and detects anxiolytic-like or anxiogenic-like agents. The open field area is made of white acrylic (40 cm diameter and 30 cm wall height). The floor had eight squares of equal area. After 60 minutes of treatment with 10 mL/kg vehicle (10% DMSO 10 mL/kg p.o.), LQFM-021 (15, 30 and 60 mg/kg, p.o.) or after 30 minutes of treatment with morphine (5 mg/kg s.c.), animals (n = 8) were placed individually at the center of the open field and observed for 5 min. The exploratory activities of animals were registered: square invaded, square invaded and time spent in the periphery, rearings, grooming, immobility and fecal boluses. The results are expressed as the means ± SEM as described by Archer [31].

2.6.3. Pentobarbital-induced sleeping test

This sleeping test is used to detect agents with sedative/depressant or stimulant effects, stimulant drugs reduce sleeping time and depressant drugs increase this time. After 60 minutes of treatment with 10 mL/kg vehicle (10% DMSO 10 mL/kg p.o.), LQFM-021 (15, 30 and 60 mg/kg, p.o.) or after 30 minutes of treatment with morphine (5 mg/kg s.c.), all groups of mice (n = 8) were treated with sodium pentobarbital (40 mg/kg i.p. – hypnosis inducer). The time elapsed between the loss and subsequent recovery of the righting reflex was taken as the latency (s) and sleeping duration (min). The results are expressed as the means ± SEM of the latency and sleeping duration as described by Carlini and Burgos [32].

2.7. Statistical analysis

The data were analyzed statistically by One-way ANOVA, followed by Tukey's test as the post hoc test or Two-way ANOVA, followed by post-hoc Bonferroni Test [33]. All statistical analyses were carried out using GraphPad InStat® version 5.00. Values of $P \leq 0.05$ are considered significant.

3. Results

3.1. Antinociceptive analysis

3.1.1. Writhing test

In the acetic acid-induced writhing test, the treatment with LQFM-021 at the tested doses of 17, 75 and 300 mg/kg decreased the number of writhing, when compared to the control group of 61.5 ± 5.3 to 32.3 ± 6.2, 29.7 ± 4.7 22.1 ± 2.3, respectively. The group treated with indomethacin (10 mg/kg p.o.), positive control, also decreased significantly the number of writhings 26.1 ± 2.8 (Fig. 2).

3.1.2. Formalin test

In the first phase of the formalin test, animals treated with vehicle showed the licking time (s) of 74.00 ± 3.02 and in the second phase of 177.6 ± 22.9. The treatment with LQFM-021 15, 30 and 60 mg/kg showed significant antinociceptive activity when compared to the control group in both phases of this test (in the first phase:

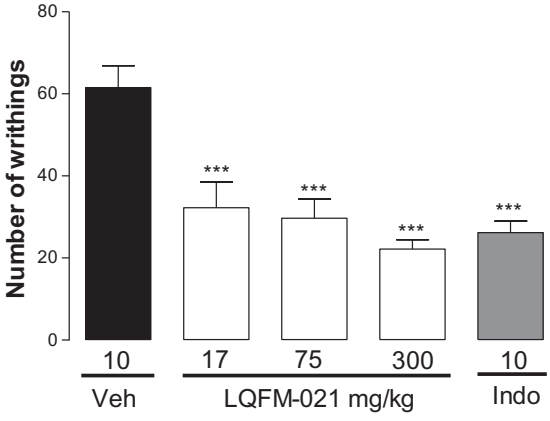

Fig. 2. Effect of LQFM-021 (17, 75 and 300 mg/kg p.o.) on the number of acetic acid-induced writhing in mice (n = 8). Vehicle (Veh; 10% DMSO 10 mL/kg p.o.). Indomethacin (Indo, 10 mg/kg p.o.) was used as positive control. Vertical bars represent mean ± SEM of number of writhings in 30 min for each experimental group. *** $p \leq 0.001$ according to ANOVA followed by post-hoc Tukey's test.

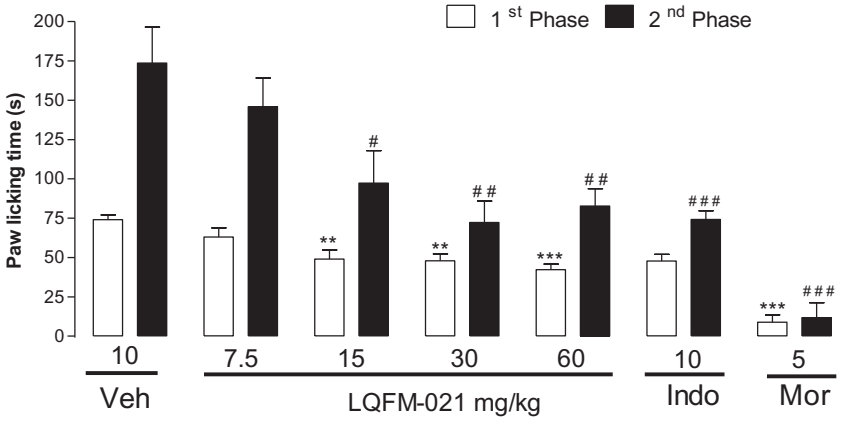

Fig. 3. Effect of LQFM-021 (7.5, 15, 30 and 60 mg/kg p.o.) on the formalin test in mice (n = 8). Indomethacin (10 mg/kg p.o.) and morphine (5 mg/kg s.c.) on the licking time (s) were used as positive control of test, during the first (0–5 min) and second phase (15–30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. **p ≤ 0.01 and *** p ≤ 0.001(compared with control group) and # p ≤ 0.05, # # p ≤ 0.01 e # # # p ≤ 0.001 (compared with second phase control group) according to ANOVA followed by post-hoc Tukey's test.

49.00 ± 5.8, 47.9 ± 4.4 and 42.7 ± 3.6, respectively, and in the second phase: 97.3 ± 20.6, 72.4 ± 13.7 and 82.7 ± 11.1, respectively). The group treated with indomethacin (10 mg/kg p.o.), anti-inflammatory positive control, decreased significantly the licking time (s) only in the second phase (74.1 ± 5.5). However, morphine (5 mg/kg s.c.), antinociceptive positive control, decreased significantly in both phases of this test (first phase: 8.87 ± 4.46 and second phase: 11.8 ± 9.5). (Fig. 3).

3.1.3. Tail flick test

In the thermal nociception test, tail-flick, the treatment with LQFM-021 (30 mg/kg, p.o.) manifested no significant antinociceptive activity when compared to the control group (vehicle), the morphine (5 mg/kg, s.c. – opioid agonist) demonstrated significant antinociception at the time of 30, 60, 90, 120 and 150 min after treatment (Fig. 4).

3.1.4. Hot plate test

In the thermal nociception test, hot plate, the treatment with LQFM-021 (30 mg/kg, p.o.) manifested no significant antinociceptive activity when compared to the control group (vehicle), the morphine (5 mg/kg, s.c. – opioid agonist) demonstrated significant antinociception at the time of 30, 60, 90 and 120 min after treatment (Fig. 5).

3.2. Analysis of the possible mechanism of LQFM 021 action

3.2.1. Involvement of opioid receptors

The administration of the non-selective opioid receptor antagonist naloxone (3 mg/kg, i.p.), given 15 min prior to test, affected the antinociceptive activity produced by LQFM-021 30 mg/kg in both phases of the formalin test. The administration of naloxone alone, in the dose tested, did not affect formalin-induced nociception (Fig. 6).

3.2.2. Involvement of NO pathway

The involvement of the NO pathway was analyzed with the pretreatment of the mice with *L*-name, NO synthase (NOS) inhibitor (10 mg/kg, given 15 min prior to test). When *L*-name was administered *per se*, in the dose tested, it was not notably different from the control group. This dose was able to significantly reverse the antinociceptive activity exhibited by LQFM-021 (30 mg/kg p.o.) in

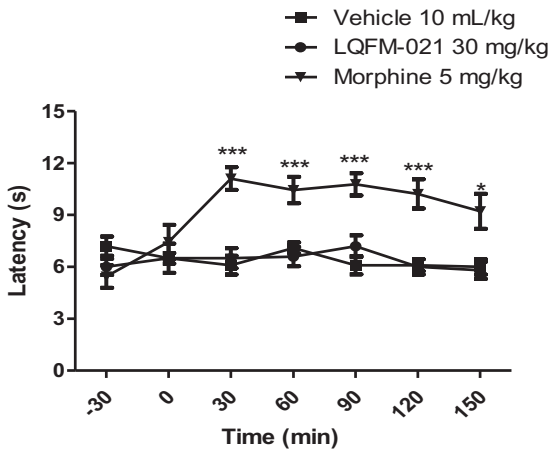

Fig. 4. Effect of LQFM-021 (30 mg/kg p.o.), and morphine (5 mg/kg s.c. – positive control) on the tail-flick test, in mice (n = 8). The values are expressed as mean ± SEM of the latency for the nociceptive behavior, in seconds. *p ≤ 0.05 and *** p ≤ 0.001 (compared with control group) according to two-way ANOVA followed by post-hoc Bonferroni test.

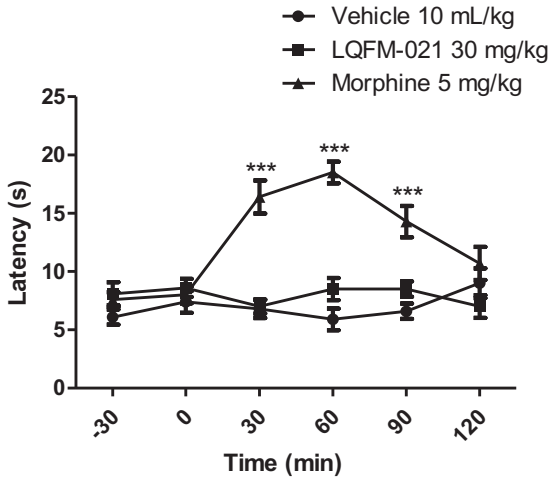

Fig. 5. Effect of LQFM-021 (30 mg/kg p.o.), and morphine (5 mg/kg s.c. – positive control) on the hot plate test, in mice (n = 8). The values are expressed as mean ± SEM of the latency for the nociceptive behavior, in seconds. *** p ≤ 0.001 (compared with control group) according to two-way ANOVA followed by post-hoc Bonferroni Test.

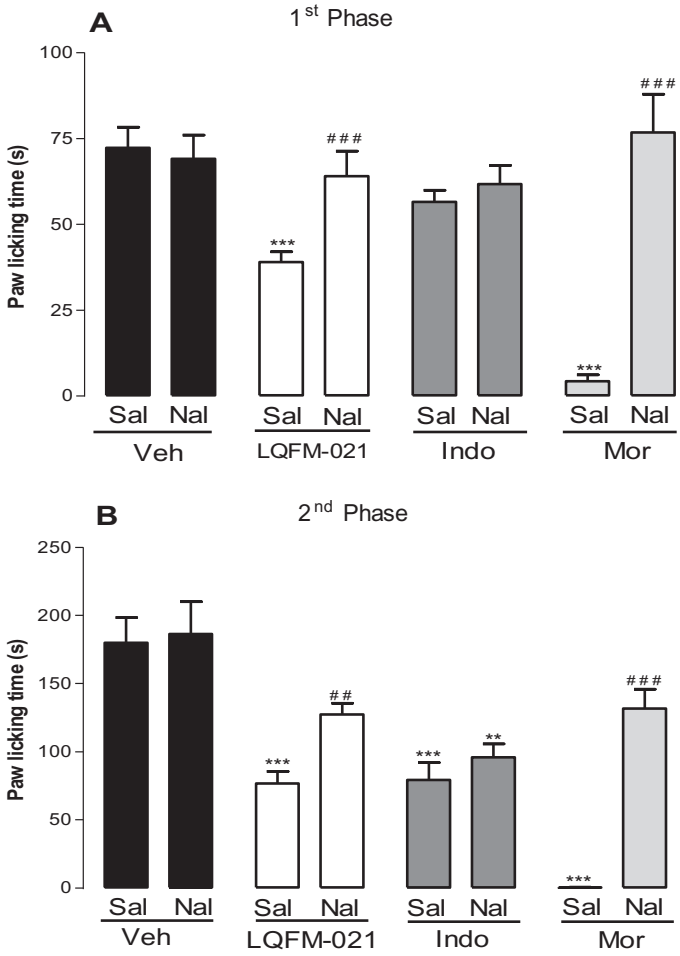

Fig. 6. Effect of pre-treatment with saline (10 mL/kg i.p.) or the non-selective opioid receptor antagonist naloxone (3 mg/kg i.p.) on the LQFM-021 (30 mg/kg p.o.) antinociceptive effect in the first phase (A) and second phase (B) of the formalin test, in mice (n = 8). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001 (compared with control group) and # # p ≤ 0.01 and # # # p ≤ 0.001 (compared with respectively treated group) according to ANOVA followed by post-hoc Tukey's test. Abbreviations: Indo: indomethacin; Nal: naloxone; Sal: saline.

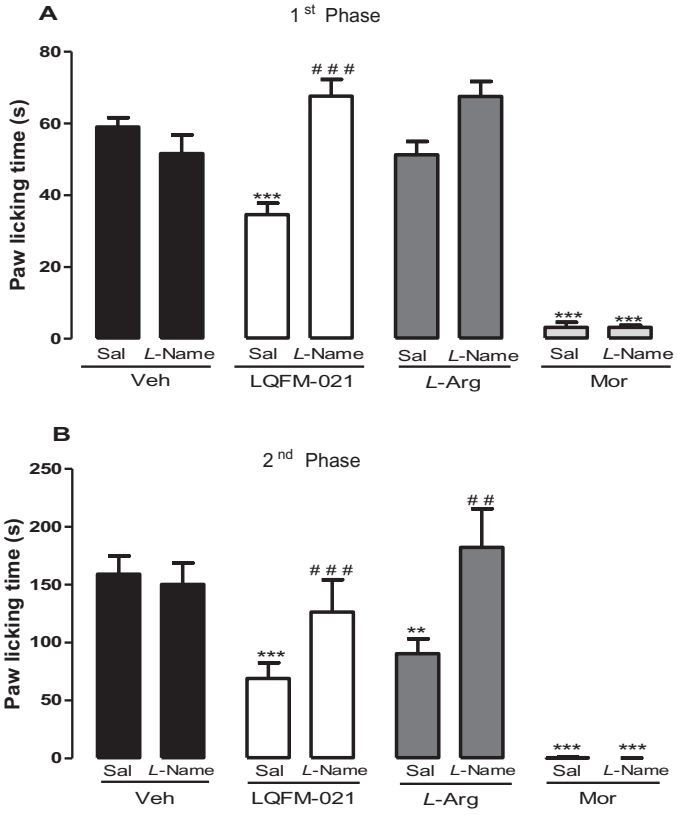

Fig. 7. Effect of pre-treatment with saline (10 mL/kg i.p.) or *L*-Name (10 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg p.o.), *L*-arginine (600 mg/kg, i.p.) and morphine (5 mg/kg s.c.) antinociceptive effect in the first phase (A) and second phase (B) of the formalin test, in mice (n = 8). Vertical bars represent mean ± SEM of pain reaction time, in seconds. Vertical bars represent mean ± SEM of pain reaction time, in seconds. ** p ≤ 0.01 and *** p ≤ 0.001 (compared control group) and # # p ≤ 0.01 e # # # p ≤ 0.001 (compared with respectively treated group) according to ANOVA followed by post-hoc Tukey's test. Abbreviations: Sal: saline; Veh: vehicle; *L*-Arg: *L*-arginine; Mor: morphine.

both phases. The *L*-name in the dose did not reverse the antinociceptive effect of the morphine (5 mg/kg s.c.) in both phases, but reverse the effect of the *L*-arginine (600 mg/kg i.p.), nitric oxide precursor, in the second phase (Fig. 7).

3.2.3. Involvement of cyclic guanosine monophosphate (cGMP) pathway

The intraperitoneal administrations of ODQ (1 mg/kg i.p.) given 15 min prior to the test, significantly reversed the antinociceptive effect induced by LQFM-021 in both phases of the formalin test. The administration of ODQ *per se*, in the dose tested, did not affect formalin-induced nociception (Fig. 8).

3.2.4. Involvement of ATP-sensitive K^+ channel pathway

The intraperitoneal administrations of glibenclamide (3 mg/kg, i.p.) given 15 min prior to the test, significantly reversed the antinociceptive effect induced by LQFM-021 in both phases of the formalin test. The administration of glibenclamide *per se*, in the dose tested, did not affect formalin-induced nociception (Fig. 9).

3.3. Behavioral alterations

3.3.1. Chimney test

The treatment with LQFM-021 (15, 30 and 60 mg/kg) did not induce impairment of motor coordination in the chimney test; while with morphine (5 mg/kg) an impairment of motor coordination was observed (Table 1).

3.3.2. Open field test

LQFM-021, at the doses used in the nociception tests (15 and 30 mg/kg), did not alter significantly the parameters observed in the open field test. Only the dose of 60 mg/kg increased the total locomotion, peripheral locomotion (squares crossed and time in the periphery). On the other hand, morphine increased the animals' exploratory activity and altered other parameters of the open field (Table 2).

3.3.3. Pentobarbital-induced sleep test

Treatment with LQFM-021 (30 and 60 mg/kg) significantly decreased sleep latency (s) from 157.4 ± 4.7 to 137.9 ± 5.5 and 121.9 ± 3.8, respectively; only the dose of 60 mg/kg increased significantly the sleeping time (min) to 96.6 ± 6.5 when compared to the control group of 55.8 ± 5.15, while morphine did not alter latency time but increased sleeping duration to 85.4 ± 6.7 min (Table 1).

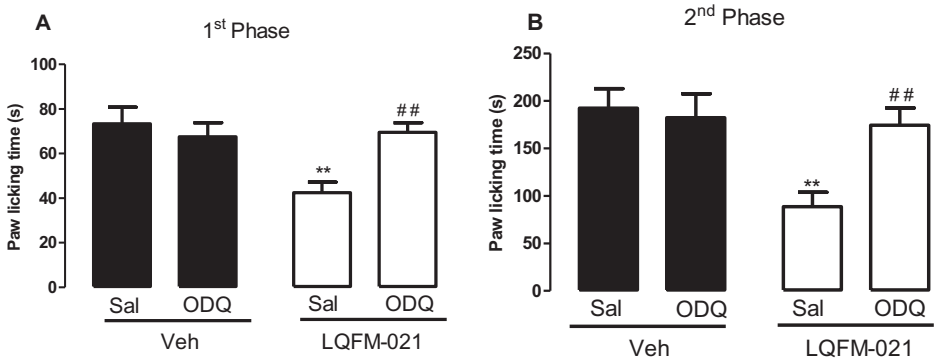

Fig. 8. Effect of pre-treatment with saline (10 mL/kg i.p.) or soluble cGMP inhibitor ODQ (10 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg p.o.), antinociceptive effect in the first phase (A) and second phase (B) of the formalin test, in mice (n = 8). Vertical bars represent mean ± SEM of pain reaction time, in seconds. Vertical bars represent mean ± SEM of pain reaction time, in seconds. **p ≤ 0.01 (compared control group) and # # p ≤ 0.01 (compared with respectively treated group) according to ANOVA followed by post-hoc Tukey's test. Abbreviations: Sal: saline; Veh: vehicle.

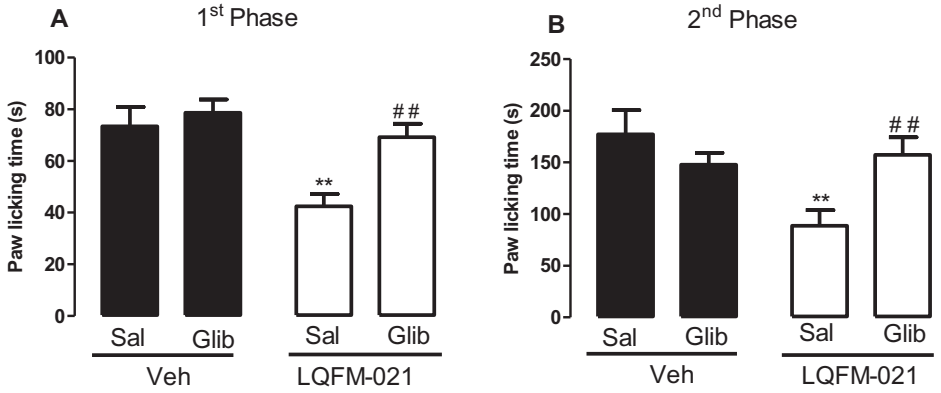

Fig. 9. Effect of pre-treatment with saline (10 mL/kg i.p.) K_{ATP} channel blocker glibenclamide (3 mg/kg i.p.) given 15 min prior to test, on the LQFM-021 (30 mg/kg p.o.), antinociceptive effect in the first phase (A) and second phase (B) of the formalin test, in mice (n = 8). Vertical bars represent mean ± SEM of pain reaction time, in seconds. Vertical bars represent mean ± SEM of pain reaction time, in seconds. **p ≤ 0.01 (compared control group) and # # p ≤ 0.01 (compared with respectively treated group) according to ANOVA followed by post-hoc Tukey's test. Abbreviations: Sal: saline; Veh: vehicle; Glib: glibenclamide.

Table 1
Effects of LQFM-021 and morphine on chimney and pentobarbital-induced sleep tests. Results are expressed as mean ± SEM of groups of mice (n = 8) treated with vehicle 10 mL/kg, p.o. (Control), LQFM-021 (15, 30 and 60 mg/kg, p.o.) or morphine (5 mg/kg s.c.). * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001, compared with control group using ANOVA followed by post-hoc Tukey's test.

Treatments	Chimney	Sleeping test	
	Time (s)	Latency (s)	Duration (min)
Vehicle 10 mL/kg	4.1 ± 0.41	157.5 ± 4.74	55.8 ± 5.15
LQFM-021 15 mg/kg	5.5 ± 0.53	138.1 ± 6.48	57.2 ± 4.70
LQFM-021 30 mg/kg	5.7 ± 0.65	137.9 ± 5.78*	63.6 ± 5.80
LQFM-021 60 mg/kg	4.6 ± 0.65	121.9 ± 3.83***	96.6 ± 6.50***
Morphine 5 mg/kg	8.8 ± 1.3 **	159.8 ± 5.76	85.4 ± 6.70***

4. Discussion

Pain is a health problem, and there is a need for new drugs that provide effective and safe treatment of pain [34]. Since previous studies have shown that pyrazole derivatives have antinociceptive activity with limited adverse effects, these compounds are an interesting source for novel analgesic drugs [21,35–37].

The pyrazole derivative LQFM-021 was originally designed through molecular hybridization from milrinone and cilostazol. The authors showed by computational molecular docking analysis which LQFM-021 is a possible inhibitor of PDE-3. In addition, the authors also viewed this pyrasole induced the relaxation of isolated arteries to alter the flow of K^+ and Ca^{2+} through the cell membrane. In addition LQFM 021 was well tolerated when administered orally [22].

Table 2
Effect of LQFM-021 and morphine on the open field test. Results are expressed as mean ± SEM of groups of mice (n = 8) treated with vehicle 10 mL/kg, p.o. (Control), LQFM-021 (15, 30 and 60 mg/kg, p.o.) or morphine (5 mg/kg s.c.). * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001, compared with control group using ANOVA followed by post-hoc Tukey's test.

Treatments	Total square crossed	Square crossed in the periphery	Time spent in the periphery (s)	Rearing	Grooming	Immobility (s)	Fecal boluses
Vehicle 10 mL/kg	124.5 ± 7.64	61.3 ± 4.69	161.9 ± 7.48	66 ± 4.77	2.11 ± 0.77	9.88 ± 2.16	1.3 ± 0.3
LQFM-021 15 mg/kg	131.8 ± 7.54	63.4 ± 4.08	186.2 ± 954	74.5 ± 3.91	1.63 ± 1.17	6.70 ± 2.02	1.5 ± 0.5
LQFM-021 30 mg/kg	133.5 ± 5.66	71.2 ± 6.06	183.0 ± 10.37	69.4 ± 2.24	2.00 ± 0.91	6.44 ± 1.92	1.5 ± 0.52
LQFM-021 60 mg/kg	158.3 ± 8.80*	89.3 ± 8.12*	213.9 ± 14.65*	78.3 ± 8.95	2.80 ± 1.24	9.74 ± 3.80	1.6 ± 0.45
Morphine 5.0 mg/kg	152 ± 8.91*	90.1 ± 2.34**	194.7 ± 5.74*	47.75 ± 13.06*	0.52 ± 0.30**	8.5 ± 2.46	0.0 ± 0.0***

Several studies reported evidence that nitric oxide (NO) and cGMP have an important role in peripheral antinociception induced by morphine [4,9], dipyrone [10], diclofenac [11], xylazine [12], acetylcholine [38] and rofecoxib [39]. The NO is a controversial neuromediator in nociception. It is able to produce pro-nociceptive or antinociceptive effects for central or peripheral pain mechanisms [38]. Duarte et al. [5] have proposed that the *L*-arginine/NO/cGMP pathway was involved in peripheral antinociception.

The present study demonstrates that LQFM-021 is able to produce antinociceptive effects in the acetic acid-induced writhing test and both phases of the formalin test. The administration of the acetic acid induced writhing which induced acute peripheral inflammatory reaction, this a visceral pain model that is widely used to evaluate antinociceptive activity [21,40]. The treatment with LQFM-021 caused a significant decrease in the number of acetic acid-induced abdominal writhing. Based on a similar effect observed with all doses, we decided to work with lower doses in other tests.

The formalin administration causes a biphasic response. The first phase (neurogenic phase) occurs during the first 5 min after formalin injection and the second phase (inflammatory phase) occurs during the 15–30 minutes after formalin injection [41,42]. This is a nociception test well described and the pain can be inhibited by typical analgesic (both phases) and anti-inflammatory drugs (second phase) [24,41]. In this test the results showed that LQFM-021 at 15 mg/kg caused significant antinociception in both phases of neurogenic and inflammatory pain. The result found did not discriminate the anti-inflammatory activity of the central or peripheral analgesic effect.

We further assessed the effects of LQFM-021 using the tail-flick and hot-plate tests; both are based on measuring the response of the animal to thermal stimuli where the tail-flick monitors a spinal reflex, and the hot plate is used for supraspinal reflex [41,43]. The treatment of the animals with LQFM-021 30 mg/kg (p.o.) did not increase latency in these tests; it was shown that the antinociceptive effect found in others tests do not involve central mechanisms. The reference drug morphine, an opioid receptor agonist, induced a significant increase in latency, as expected.

Although centrally mediated antinociceptive effect was excluded, we decided to investigate the participation of opioid receptor in the mechanism of LQFM-021's action. Since which opioid receptors on peripheral terminals of afferent nerves can be the sites of the intrinsic modulation of nociception and drugs with action on these receptors may lead to analgesic effects in the absence of the central adverse effects caused by opioids [44].

The formalin test was used to clarify the possible mechanism of the antinociceptive effect of the compound. This study showed that the administration of LQFM-021 (30 mg/kg) produced antinociceptive effects on both the neurogenic and inflammatory phases of the formalin test, and the pre-treatment with naloxone (3 mg/kg), opioid receptor antagonist, completely reversed the effect produced by LQFM-021 in the first phase and partly in the second phase. These results indicate that this compound has peripheral antinociceptive activity that involves activation of the opioid receptor. A similar result was found in the study of the other pyrasole by Prokopp et al. [19]. Some studies have demonstrated that peripheral opioid receptors induce peripheral antinociception activating the *L*-arginine/NO/cGMP pathway [4,8,44].

The pre-treatment with the NO synthesis inhibitor, *L*-NAME, completely reversed the antinociceptive effects of LQFM-021 in the both phases of the formalin test. These data support the hypothesis that LQFM-021 could produce an peripheral antinociceptive effect through the *L*-arginine/NO/cGMP signaling pathway similar to other analgesic drugs.

The NO/cGMP pathway depends on the synthesis and release of NO which then activates the guanylate cyclase enzyme responsible for an increase in the intracellular level of the cGMP [12]. It has been reported that cGMP plays an important role in the up or downregulation of nociceptors and is a key mediator in antinociception [4,45,46]. The pre-treatment with the specific guanylate cyclase inhibitor, ODQ, reversed the antinociceptive effect of LQFM-021 in both phases of the formalin test, as shown by the involvement of cGMP in its effect. Our results are agree with several studies showing that peripheral NO and cGMP are important for the antinociceptive activity of different analgesic drugs [5,6,14,47,48]. Furthermore, substances that inhibit cGMP hydrolysis could increase the activity of substances that signal cGMP pathway, such as inhibitor of the phosphodiesterase [49,50].

Therefore, due to the hypothesis that activation of the NO/cGMP pathway could induce antinociception through the opening of K^+ channels [9], we researched on the possible involvement of ATP-sensitive K^+ channels in peripheral antinociception induced by LQFM-021. The sulfonylurea glibenclamide (specific K^+_{ATP} blocker) inhibits the antinociceptive effect induced by a NO donor, sodium nitroprusside, and an analog of cGMP, dibutyryl-cGMP [51,52]. Our results demonstrated that the pre-treatment with the glibenclamide antagonized the antinociceptive effect of LQFM-021 in both phases of the formalin test. Taken together, these data suggest that LQFM-021 produces a peripheral antinociceptive effect this compound involved the participation of opioid receptors and NO/cGMP/K_{ATP} pathway.

In addition, to clarify if the analgesic effect is not a result of motor deficits or sedation, mice were subjected to chimney, open-field and pentobarbital-induced sleep tests after oral administration of LQFM-021. Our results showed that the treatment with LQFM-021, in analgesic doses, did not cause an impairment of locomotor activity in the chimney test. Only the higher dose used in the study of antinociceptive activity promoted changes in the open field and pentobarbital-induced sleep tests. This result suggested that the antinociceptive effect of LQFM-021 was not false positive with peripheral neuromuscular blockade or sedation.

5. Conclusions

In conclusion, this study showed that new pyrazole compound LQFM-021 produced antinociceptive effects in the acetic acid-induced writhing and formalin tests. However results of tests of thermal pain indicate that its action is only peripheral. In addition, this study suggests that the mechanism of antinociceptive action involved interaction with peripheral opioid receptors and activation of the NO/cGMP/$K_{(ATP)}$ pathway.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.niox.2015.02.146.

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